

Intrinsic Phenotypic Differences of Asthmatic Epithelium and Its Inflammatory Responses to Respiratory Syncytial Virus and Air Pollution

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A substantial proportion of healthcare cost associated with asthma is attributable to exacerbations of the disease. Within the airway, the epithelium forms the mucosal immune barrier, the first structural cell defense against common environmental insults such as respiratory syncytial virus (RSV) and particulate matter. We sought to characterize the phenotype of differentiated asthmatic-derived airway epithelial cultures and their intrinsic inflammatory responses to environmental challenges. Air-liquid interface (ALI) cultures were generated from asthmatic ($n = 6$) and nonasthmatic ($n = 6$) airway epithelial cells. Airway tissue and ALI cultures were analyzed by immunohistochemistry for cytokeratin-5, E-cadherin, Ki67, Muc5AC, NF- κ B, the activation of p38, and apoptosis. ALI cultures were exposed to RSV (4×10^6 plaque forming unit/ml), particulate matter collected by Environmental Health Canada (EHC-93, 100 μ g/ml), or mechanically wounded for 24, 48, and 96 hours and basolateral supernatants analyzed for inflammatory cytokines, using Luminex and ELISA. The airway epithelium in airway sections of patients with asthma as well as *in vitro* ALI cultures demonstrated a less differentiated epithelium, characterized by elevated numbers of basal cells marked by the expression of cytokeratin-5, increased phosphorylation of p38 mitogen-activated protein kinase, and less adherens junction protein E-cadherin. Transepithelial resistance was not different between asthmatic and nonasthmatic cultures. In response to infection with RSV, exposure to EHC-93, or mechanical wounding, asthmatic ALI cultures released greater concentrations of IL-6, IL-8, and granulocyte macrophage colony-stimulating factor, compared with nonasthmatic cultures ($P < 0.05$). This parallel *ex vivo* and *in vitro* study of the asthmatic epithelium demonstrates an intrinsically altered phenotype and aberrant inflammatory response to common environmental challenges, compared with nonasthmatic epithelium.

Keywords: airway epithelium; asthma; inflammation and repair; RSV; particulate matter

(Received in original form January 27, 2011 and in final form May 24, 2011)

This work was supported by Allergen-National Centre for Excellence/Canadian Institutes for Health Research grant 79632. T.-L.H. is a recipient of a CIHR/Canadian Lung Association/GlaxoSmithKline, Integrated and Mentored Pulmonary and Cardiovascular Training strategic training, and a Michael Smith Foundation for Health Research postdoctoral fellowship. D.A.K. is a Canada Research Chair in Airway Disease and Michael Smith Foundation for Health Research Career Investigator. D.R.D. is a CIHR New Investigator and Michael Smith Foundation for Health Research Career Investigator. S.V.E. is a senior scholar with the Michael Smith Foundation for Health Research and a CIHR/GSK Professor in Chronic Obstructive Pulmonary Disease.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 45, pp 1090–1100, 2011

Originally Published in Press as DOI: 10.1165/rcmb.2011-0031OC on June 3, 2011

Internet address: www.atsjournals.org

Asthma remains a heavy burden on healthcare systems, despite advances in our understanding of its pathogenesis. In particular, the costs related to hospital admissions during asthma exacerbations constitute a substantial proportion of the financial burden associated with the disease (1). Epidemiological studies suggest that exposure to urban particulate matter promotes the development of asthma (2), and is associated with exacerbations of asthma (3–5). Many studies demonstrated that acute increases in air pollution result in a greater use of asthma medications and increased hospital admissions for asthma (6–8). Similarly, viral respiratory tract infections, particularly with respiratory syncytial virus (RSV) or rhinovirus, are most commonly associated with exacerbations of asthma (80% in children and 50–76% in adults) (9–12). Accordingly, to understand how these environmental exposures exacerbate the underlying mechanisms of asthma is important.

The epithelium is the major target for both RSV infections (13–15) and inhaled airborne particles (16–18). In asthma, abundant evidence exists that the epithelium is structurally abnormal, with well-documented evidence of epithelial damage and denudation (19). Indeed, the asthmatic epithelium is widely accepted to be fragile and to display a loss of columnar ciliated cells in both adults (20) and children (21). This is coincident with a reduced and altered expression of adhesion proteins such as E-cadherin and zonula occludens-1 (22). Epithelial damage is not only confined to the lower airways, as disrupted formation of desmosomes has been shown in nasal polyps from children with asthma (23). The composition of asthmatic airway epithelium itself, compared with nondiseased airways, has also been demonstrated to be different, with an increased expression of the basal epithelial marker cytokeratin-5 and of p63 in both pediatric and adult patients with asthma (24, 25). Goblet-cell hyperplasia and excessive mucus production are also common features of asthma.

These morphological changes are mirrored at the molecular level in cultured asthmatic epithelial cells, which are more susceptible to oxidant-induced “stress,” and which display an abnormal expression of proinflammatory transcription factors (NF- κ B, activator protein 1 factor, signal transducer and activator of transcription [STAT]–1, and STAT6) as well as heat-shock proteins (26–28). In the asthmatic epithelium, the expression of epithelial growth factor receptor (EGFR) is markedly increased, especially in areas where columnar cells have been shed in both adults (29) and children with moderate/severe asthma (30). The expression of the cyclin-dependent kinase inhibitor p21WAF1/CIP1 is also increased in the asthmatic epithelium (31), which may explain the apparent discrepancy between concentrations of EGFR and the proliferative response. Recently, asthmatic epithelial cells were shown to display attenuated wound repair *in vitro*, as well as a concurrent elevation of plasminogen activator inhibitor 1 expression (32) and decreased expression of

fibronectin (33). Thus, the available evidence suggests that the asthmatic epithelium is fragile and contains an altered phenotype of epithelial cells, which could occur because of excessive damage or a compromised ability to differentiate.

However, evidence of functional abnormalities in the pseudostratified asthmatic epithelium is not as clear, especially in response to environmental exposures such as RSV and particulate matter. The objectives of this study were thus to: (1) determine if structural abnormalities in airway sections could also be modeled in pseudostratified air-liquid interface (ALI) cultures, and (2) determine if functional differences in the inflammatory response exist between asthmatic and nonasthmatic ALI cultures in response to RSV and particulate matter exposure.

MATERIALS AND METHODS

Airway Epithelial Cell Isolation and Culture

De-identified asthmatic and nonasthmatic donor lungs, not suitable for transplantation and donated to medical research, were obtained through the International Institute for the Advancement of Medicine (Edison, NJ). Epithelial cells were isolated by protease digestion, as previously described (34). Donor deaths were primarily attributable to head trauma in nonasthmatic patients, whereas five of six patients with asthma were thought to have died during exacerbations of their asthma. Subject demographics and clinical details are listed in Table 1. This study was approved by the Ethics Committee of the University of British Columbia. Donor airways and donor-matched ALI epithelial cultures were generated using cells at Passages 1 or 2, and analyzed using immunohistochemistry and transmission electron microscopy (TEM) (see online supplement).

Infection with RSV

ALI cultures were stimulated with or without 100 μ l of ALI media (see online supplement for methods; MatTek Corporation, Ashland, MA) containing 4×10^6 plaque forming units/ml RSV strain A2 (multiplicity of infection = 3) at the cultures' apical surface for 90 minutes, and excess media were removed. ALI cultures and basolateral supernatants were harvested at 24, 48, and 96 hours, and then fixed and embedded for immunohistochemical analysis or stored at -80°C .

Particulate Matter Challenge

Particulate matter was provided by Environmental Health Canada (EHC), and had been collected in Ottawa in 1993 from ambient air filters (termed EHC-93; Environmental Health Canada, Ottawa, Ontario, Canada). ALI cultures were stimulated with 100 μ l of 100 $\mu\text{g}/\text{ml}$ EHC-93 or ALI media at the apical surface of ALI cultures for 90 minutes, and then excess media were removed (see online supplement for details). We have previously used 100 $\mu\text{g}/\text{ml}$ of EHC-93 to represent

background exposure to air pollution (16, 35). Basolateral supernatants and ALI cultures were harvested at for 24, 48, and 96 hours, and stored at -80°C or embedded for immunohistochemical analysis.

Mechanical Wounding

ALI cultures were mechanically wounded in a cross-hatched manner, using a small rubber GUM Stimulator (Butler, Sunstar Americas, Guelph, ON, Canada) down to the ALI membrane insert, washed with PBS, and reincubated with ALI media (see online supplement for details). Basolateral supernatants and ALI cultures were harvested at 24, 48, and 96 hours and stored at -80°C , or embedded for immunohistochemical analysis.

Cytokine Analysis

A multiplex cytokine array was performed using the Human LINCplex kit (Millipore, Etobicoke, ON, Canada) for GM-CSF, IL-1 β , IL-6, IL-8, IL-10, and TNF- α in duplicate, according to the manufacturer's instructions. To confirm the Luminex results, IL-6, IL-8, and granulocyte macrophage colony-stimulating factor (GM-CSF) were measured in basolateral ALI culture supernatants in duplicate, using commercial ELISA kits (Invitrogen, Carlsbad, CA), and IL-13, using an in-house ELISA (36). All results were normalized to total ALI protein lysate (Pierce, Rockford, IL).

Statistical Analysis

All experiments were performed for each individual in the study in triplicate, and data are presented as the mean $\pm$ SEM. All results were tested for population normality and homogeneity of variance. Differences between paired data were analyzed by ANOVA, with Dunnett *post hoc* correction for comparisons of group data. $P < 0.05$ was accepted as significant. Statistical analysis was performed using commercial statistical software Prism version 4.0 (GraphPad Software, Inc., La Jolla, CA).

RESULTS

Physical Properties of Asthmatic and Nonasthmatic ALI Epithelial Cultures

Airway epithelial cells derived from both patients without asthma (Figure 1A) and patients with asthma (Figure 1B) generated differentiated, multilayered ALI cultures, with no gross histological differences between them. To assess differentiation in more detail, we used TEM. High-magnification images confirmed the formation of cilia (Figures 1C and 1E), as well as tight junctions and adherens junctions (Figures 1D and 1F) in both asthmatic and nonasthmatic cultures. As demonstrated in Figure 1G, we observed no differences in transepithelial

TABLE 1. PATIENT DEMOGRAPHICS

Gender	Age (Years)	Ethnicity	Cause of Death	Medical History	Known Medication	Subbasement Membrane Fibrosis
Female	5	Caucasian	Head trauma	None	None	None
Male	22	Caucasian	Head trauma	None	None	None
Male	23	Caucasian	Head trauma	None	None	None
Male	18	Caucasian	Head trauma	None	None	None
Male	24	Caucasian	Head trauma	None	None	None
Female	4	Hispanic	Head trauma	None	None	None
Female	8	Hispanic	Anoxia/asthma	Asthma diagnosis at age 3 years	Albuterol, montelukast	Yes
Male	11	Caucasian	Anoxia/asthma	Asthma diagnosis at age 2 years	Albuterol	Yes
Female	21	Caucasian	Tylenol overdose	Asthma diagnosis, cervical cancer	Albuterol, salmeterol/fluticasone	Yes
Female	15	Caucasian	Anoxia/asthma	Environmental allergies and asthma diagnosis	Albuterol, salmeterol/fluticasone, prednisone	Yes
Male	26	Caucasian	Anoxia and brain injury	Asthma diagnosed in childhood	Albuterol	Yes
Male	6	Caucasian	Asthma	Asthma diagnosis	Albuterol	Yes

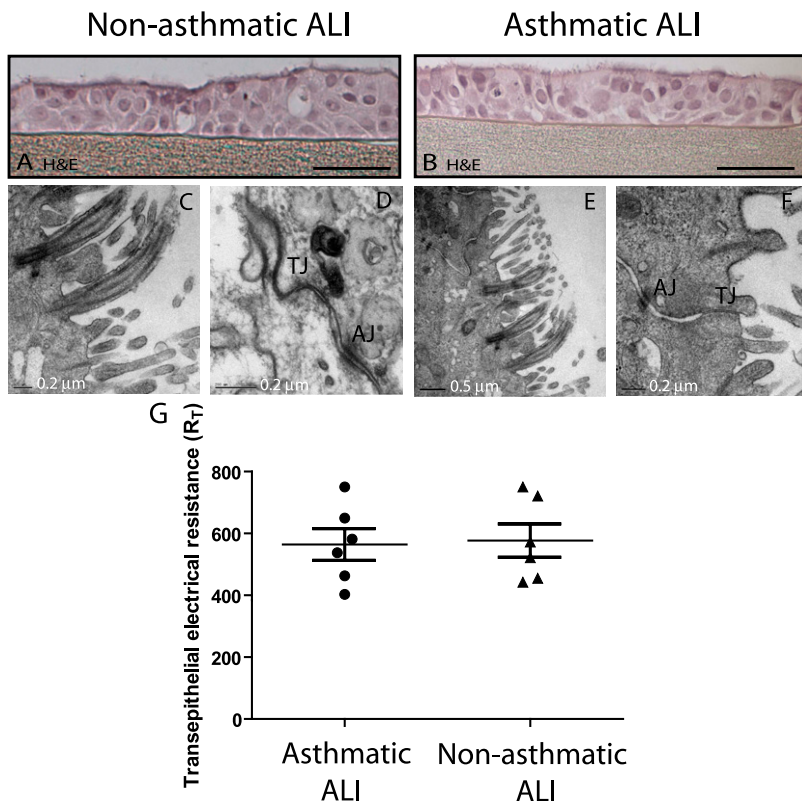


Figure 1. Generation of air-liquid interface (ALI) cultures from asthmatic and nonasthmatic airways. ALI-airway epithelial cultures derived from asthmatic (A, C, and D) and nonasthmatic donors (B, E, and F) were analyzed for morphology by hematoxylin-and-eosin-stained (H&E) sections or transmission electron microscopy for the formation of cilia (C and E) and formation of adherens junctions (AJ) and tight junctions (TJ) (D and F). Scale bar for hematoxylin-and-eosin-stained sections = 100 μ M.

electrical resistance between asthmatic and nonasthmatic cultures after 28 days of ALI culture.

Asthmatic Airway Epithelium Demonstrates an Intrinsically Altered Phenotype

To determine if the epithelial phenotype of the asthmatic airway *in vivo* is recapitulated by *in vitro* ALI cultures, we compared the immunostaining of airway sections to donor-matched ALI cultures for markers of differentiation, proliferation, apoptosis, and the production of mucus. We found that the percentage of Mucin-5AC (Muc-5AC)-positive airway epithelial cells per unit length of basement membrane was significantly elevated in the asthmatic airway compared with nonasthmatic airway sections (Figures 2A and 2B, $P < 0.05$). A similar trend was observed for periodic acid-Schiff (PAS) staining (Figures 2C and 2D). In contrast, no significant difference was evident between asthmatic and nonasthmatic-derived ALI cultures for either Muc5AC or PAS staining (Figures 2F–2I). The mean percentage of positive cells for both Muc5AC and PAS in both airways and ALI cultures for all subjects is detailed in Figure 2E.

The numbers of epithelial cells positive for the basal cell marker cytokeratin-5 were significantly elevated in asthmatic compared with nonasthmatic airway sections and ALI cultures (Figures 3A, 3B, 3J, and 3K). The expression of the proliferation marker Ki67 was increased in asthmatic airway sections compared with those derived from nonasthmatic airway sections, but this finding was not evident in ALI cultures (Figures 3C, 3D, 3L, and 3M). In contrast, the assessment of apoptosis by *in situ* oligo ligation showed only occasional apoptotic nuclei in both asthmatic and nonasthmatic cell cultures, with no statistical difference between groups (Figures 3E, 3F, 3N, and 3O). The expression of E-cadherin was decreased in asthmatic airway and ALI cultures, compared with nonasthmatic donors (Figures 3G, 3H, 3P, and 3Q). The mean percentages of positive cells per

unit length for each protein in both airway and ALI cultures, for all subjects, are shown in Figures 3I and 3R.

Asthmatic ALI Cultures Demonstrate an Enhanced Inflammatory Response to Infection with RSV

After infection with RSV for 24, 48, and 96 hours, ALI cultures were embedded and stained for RSV phosphoprotein, fusion, and nuclear proteins. As demonstrated in Figure 4A, RSV-positive cells at the apical surface of the ALI were only evident after infection, with no significant difference in the number of RSV-positive cells between asthmatic and nonasthmatic patients at the 96-hour time point (Figures 4A and 4B). We also analyzed supernatants from the basal compartment after infection. Concentrations of IL-1 β and IL-10 were below the limits of detection in all samples analyzed, and concentrations of IL-15 and TNF- α were detectable but did not change significantly after infection (data not shown). The release of IL-6, IL-8, IL-13, and GM-CSF was elevated in both asthmatic and nonasthmatic RSV-exposed ALI cultures, using Luminex (data not shown). To confirm these findings, supernatants were analyzed, using commercial ELISA kits. As shown in Figures 4C–4E, the release of IL-6, IL-8, and GM-CSF was elevated at 24, 48, and 96 hours after infection with RSV in asthmatic compared with nonasthmatic ALI cultures ($P < 0.05$). Although detectable concentrations of IL-13 were obtained using ELISA, we observed no significant difference in release after infection with RSV (Figure E1A in the online supplement).

Asthmatic ALI Cultures Demonstrate an Enhanced Inflammatory Response to EHC-93 Exposure

After incubation with EHC-93, supernatants from the basal compartment were analyzed at 24, 48, and 96 hours, using the Luminex multiplex cytokine panel. Concentrations of TNF- α and IL-15 were detectable but did not change significantly after

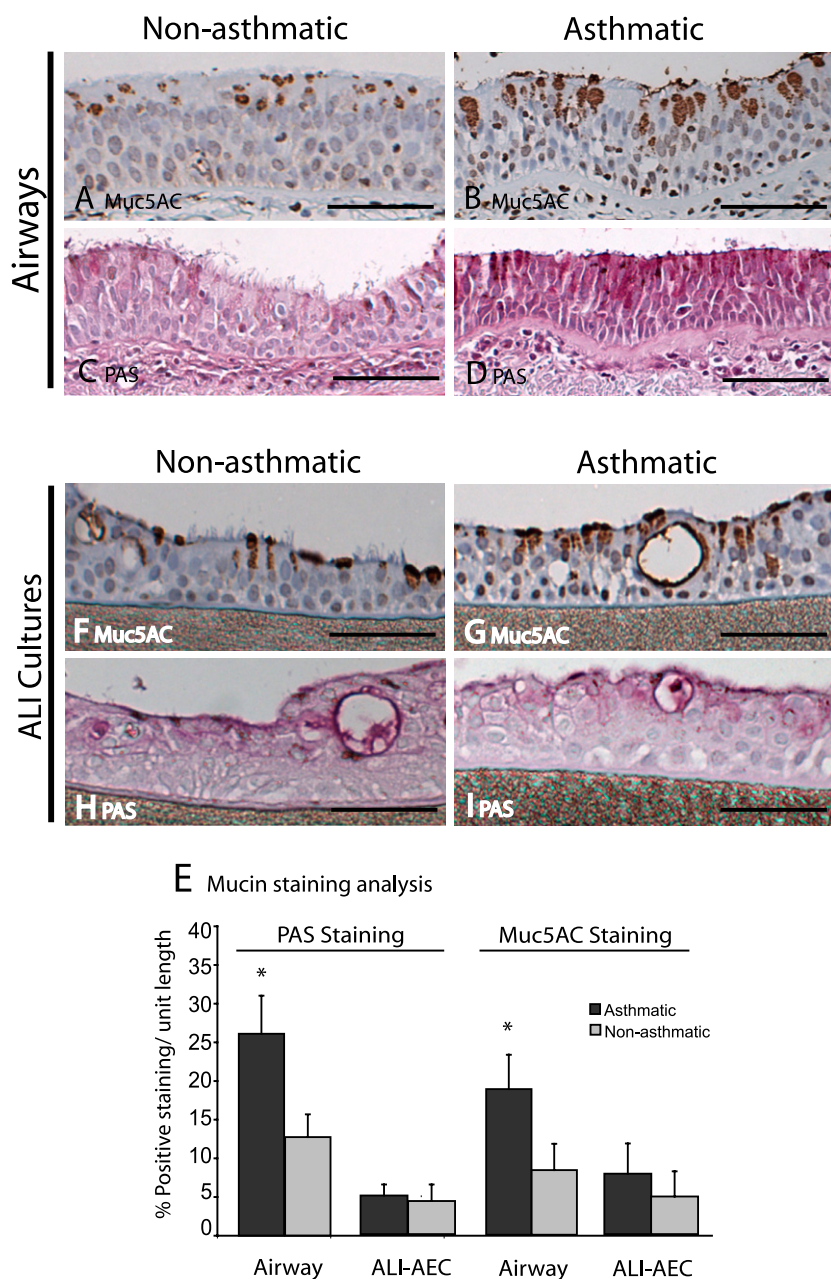


Figure 2. Expression of mucus in asthmatic epithelium. Patient-matched airway sections and ALI cultures from asthmatic ($n = 6$) and nonasthmatic ($n = 6$) donors were immunostained for Muc5AC (stained in brown) (A, B, F, and G) and periodic acid–Schiff (PAS) (stained in purple) (C, D, H, and I). (E) The expression of Muc5AC and PAS staining within the epithelium was quantified using Image Pro Plus software (Media Cybernetics, Inc., Bethesda, MD). Data are expressed as percent positive staining/unit length $\pm$ SEM. * $P < 0.05$, compared with nonasthmatic airways. Scale bar = 100 μ M.

challenge with EHC-93. IL-1 β and IL-10 were not detected in any of the samples analyzed (data not shown). Data confirmed by ELISA demonstrated that the release of IL-6 and IL-8 was elevated 24 hours after exposure to EHC-93, whereas the release of GM-CSF was slower, that is, elevated at 48 and 96 hours in asthmatic-derived ALI cultures compared with nonasthmatic ALI cultures (Figures 5A–5C). We did observe similar concentrations of basolateral IL-13 in both asthmatic and nonasthmatic ALI cultures treated with EHC-93, compared with control conditions at the initial 24-hour time point. However, this finding was not statistically significant after correcting for multiple comparisons (Figure E1B).

The Intrinsic Inflammatory Response to Mechanical Wounding

To understand further the aberrant inflammatory response generated by asthmatic epithelial cells *in vitro*, we mechanically

wounded ALI cultures to model a nonenvironmental challenge. Cultures were mechanically wounded in a cross-hatched pattern, and wound closure was calculated by manual tracing (Figure E2). Supernatants from the basal compartments of ALI cultures were analyzed using ELISA for the release of cytokines IL-6, IL-8, IL-13, and GM-CSF. Strikingly, as observed after exposure to RSV and EHC-93, we found an elevated release of IL-6 and IL-8 at 24 hours, but GM-CSF only at 96 hours, in asthmatic compared with nonasthmatic ALI cultures after mechanical wounding (Figures 6A–6C; $P < 0.05$). The release of IL-13 was also elevated to similar concentrations in both asthmatic and nonasthmatic ALI cultures after mechanical wounding, compared with nonwounded cultures at 24 hours, but this release was not statistically significant compared with control samples (Figure E1C).

The proinflammatory transcription factor NF- κ B and p38 mitogen-activated protein (MAP) kinase (p38) were previously shown to be involved in the release of cytokines from the

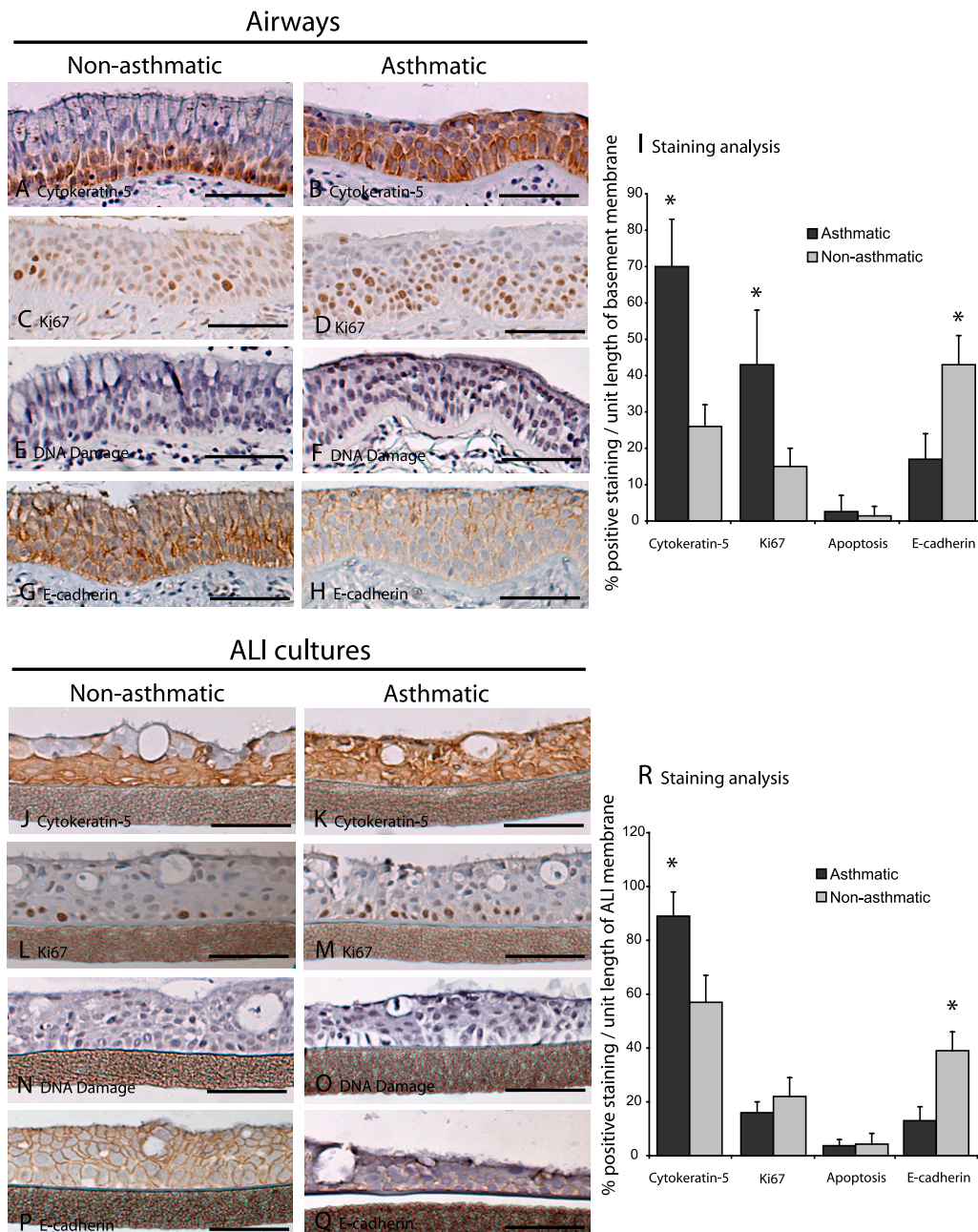


Figure 3. Asthmatic airway epithelia demonstrate an intrinsically altered phenotype. Patient-matched airway sections and ALI cultures derived from asthmatic ($n = 6$) and nonasthmatic ($n = 6$) airway epithelial cells were differentiated in culture for 28 days and then fixed, embedded in paraffin, and sectioned for immunohistochemical analysis. Sections were stained with cytokeratin-5 (A, B, J, and K), Ki67 (C, D, L, and M), *in situ* oligo ligation for apoptosis (E, F, N, and O), or E-cadherin (G, H, P, and Q). Scale bar = 100 μ m. The expression of cytokeratin-5, Ki67, apoptosis, and E-cadherin within the airway (I) and ALI cultures (R) was quantified using Image Pro Plus software. Data are expressed as percent positive staining/unit length $\pm$ SEM. * $P < 0.05$, compared with nonasthmatic epithelium.

epithelium (37–40). To understand if this intrinsic release of IL-6, IL-8, and GM-CSF cytokines was attributable to the altered activation of p38 or NF- κ B, ALI sections from 24-hour time points were stained for each protein, and the number of cells with positive nuclei was counted. Baseline levels of phospho-p38 were elevated in asthmatic ALI sections compared with nonasthmatic sections (Figure 6D; $P < 0.012$). Although we observed a trend for an increased number of phospho-p38-positive epithelial cells after exposure to RSV, EHC-93, or wounding in both asthmatic and nonasthmatic ALI cultures, this finding was not statistically significant. In contrast, the expression of nuclear NF- κ B was not different at baseline or after challenge with RSV, EHC-93, or mechanical wounding (Figure 6E).

DISCUSSION

This parallel *ex vivo* and *in vitro* study of the asthmatic epithelium demonstrates an intrinsically altered phenotype and

inflammatory response to common environmental insults, compared with normal epithelium. In particular, we demonstrate that asthmatic epithelial cells show an increased expression of the basal cell marker cytokeratin-5, the phosphorylation of p38, and a decreased expression of adherens junction protein E-cadherin. In response to commonly encountered environmental challenges such as RSV and particulate matter, or nonenvironmental challenges such as mechanical wounding, asthmatic epithelial cells exhibit an enhanced expression of IL-6, IL-8, and GM-CSF, compared with nonasthmatic epithelial cells.

We and others have previously demonstrated that ALI cultures consist of multiple cell types, including ciliated, mucous, and basal cells (25, 34, 41, 42), and as such, these cultures are advantageous over monolayer submerged cultures because they better mimic the multicellular phenotype of tracheobronchial epithelium *in vivo*. In this capacity, environmental stimuli can be applied in a more representative manner than that of submerged cultures. However, the ALI culture system does have

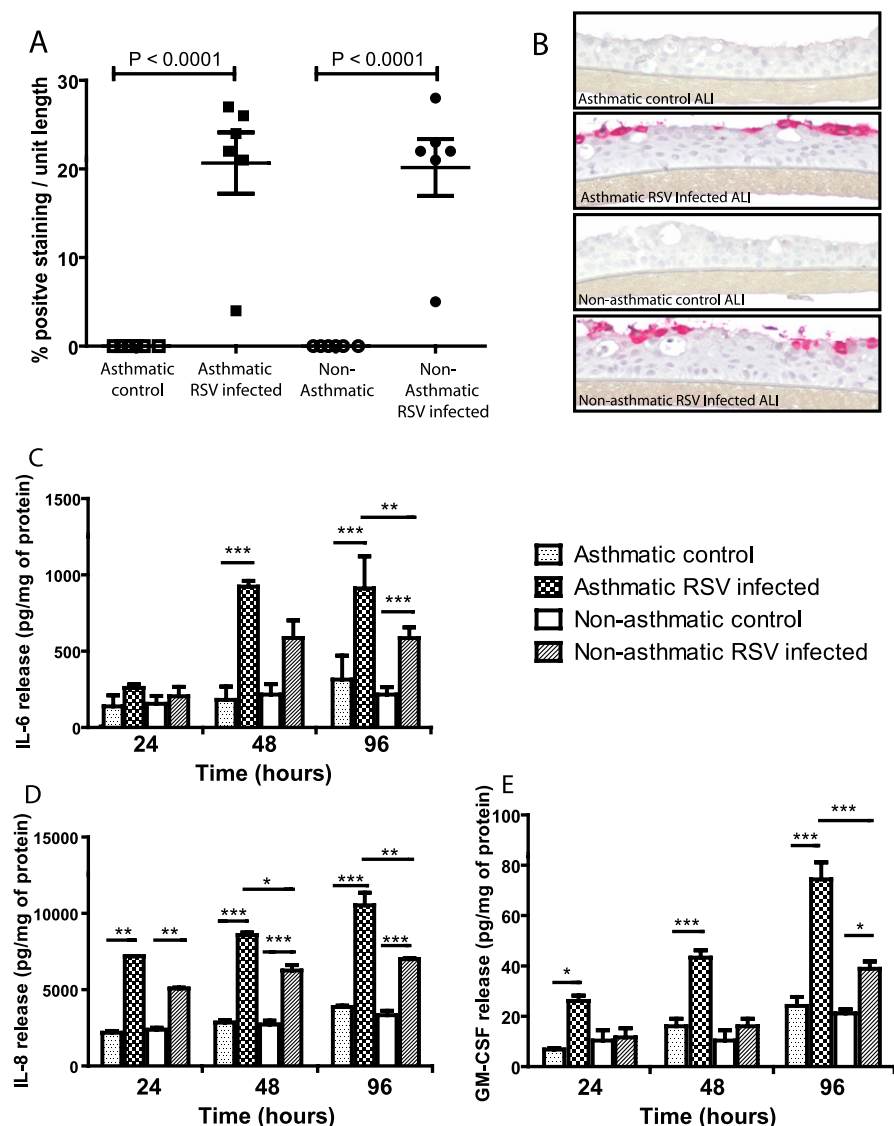


Figure 4. Asthmatic ALI cultures demonstrate an enhanced inflammatory response to infection with respiratory syncytial virus (RSV). ALI cultures derived from asthmatic and nonasthmatic airway epithelial cells were infected at the apical surface with 100 μ l of RSV-A2 at 4×10^6 plaque forming units/ml or ALI media for 24, 48, and 96 hours. (A) ALI sections were stained, and we analyzed percent positive cells/unit length. (B) RSV staining is indicated in red. Basolateral supernatants from asthmatic ALI cultures (dotted bars), RSV-treated asthmatic ALI cultures (checked bars), nonasthmatic ALI cultures (open bars), and RSV-treated nonasthmatic cultures (dashed bars) were collected and analyzed for IL-6 (C), IL-8 (D), and granulocyte macrophage colony-stimulating factor (GM-CSF) (E) by ELISA. Data are expressed for each cytokine as pg/mg of protein $\pm$ SEM. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

limitations, because ALI cultures are not pseudostratified like the epithelium of proximal airways *in vivo*. Thus it is important to note that morphological differences in the ALI culture system may influence experimental outcomes, such as epithelial repair. Specific characterizations of ALI cultures from donors with asthma are limited, and no studies, to the best of our knowledge, have focused on the effects of environmental stimuli using these models. With regard to epithelial phenotypes, we demonstrate that in asthmatic airways and matched ALI cultures, greater numbers of cells express cytokeratin-5, compared with nonasthmatic cultures. This finding is in agreement with our previous data using cells from a separate cohort of patients with asthma (25). Cytokeratin-5 is a cytoskeleton protein expressed only in basal cells, which are thought to constitute the progenitor cell of the airway epithelium (34). Parker and colleagues showed that ALI cultures derived from pediatric patients with asthma exhibited decreased numbers of ciliated cells (43). Although Parker and colleagues (43) did not specifically analyze the expression of cytokeratin-5, their findings support the notion that asthmatic cells do not differentiate appropriately. The reasons underlying the change in cytokeratin-5 expression and the phenotype of these cells in asthmatic epithelium are presently unknown, but the data support our previous findings

of increased resident epithelial progenitor cells in the asthmatic airway (34).

The epithelium of patients with asthma also manifested a reduced expression of E-cadherin, which was maintained throughout monolayer and ALI culture. Our observations are consistent with those in two studies of bronchial biopsies that demonstrated the reduced membrane expression of E-cadherin in asthmatic epithelia (20, 22). E-cadherin is essential for epithelial tissue integrity and morphogenesis through the initiation and formation of adherens junctions (44). E-cadherin has been demonstrated to modulate the multiple signaling pathways involved in epithelial repair, is key in epithelial-mesenchymal transitions, and can affect the expression of proinflammatory chemokines such as chemokine ligand 17 (45–47). The knockdown of E-cadherin expression by small interfering RNA was also shown to decrease epithelial resistance in bronchial epithelial monolayers (48). In our ALI cultures, no differences were evident in trans-epithelial resistance between asthmatic and nonasthmatic donor cultures. Several conflicting studies concerned differences in airway permeability *in vivo* between asthmatic and normal subjects (49, 50). However, our data are supported by the findings of Parker and colleagues, who also found no difference in trans-epithelial resistance between asthmatic and nonasthmatic ALI

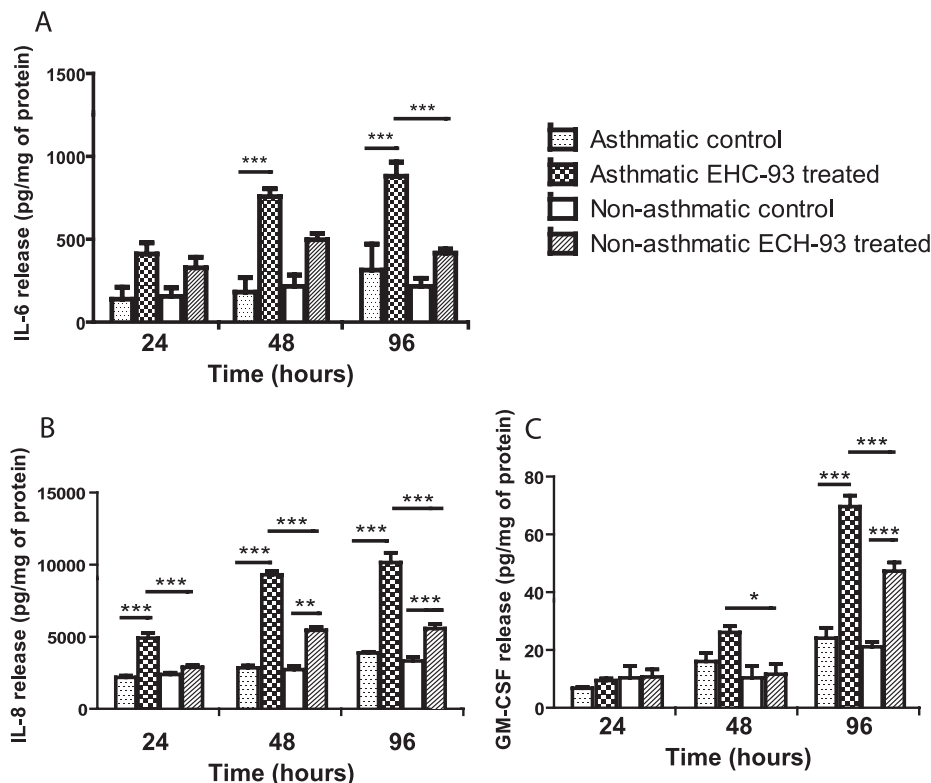


Figure 5. Asthmatic ALI cultures demonstrate an enhanced inflammatory response to particulate matter collected by Environmental Health Canada (EHC-93). ALI cultures derived from asthmatic and nonasthmatic airway epithelial cells were treated at the apical surface with 100 μ l of 100 μ g/ml EHC-93 or ALI media for 24, 48, and 96 hours. Afterward, basolateral supernatants from asthmatic ALI cultures (dotted bars), EHC-93-treated asthmatic ALI cultures (checked bars), non-asthmatic ALI cultures (open bars), and EHC-93-treated nonasthmatic cultures (dashed bars) were collected and analyzed for the release of IL-6 (A), IL-8 (B), and GM-CSF (C), using ELISA. Data are expressed for each cytokine as pg/mg of protein $\pm$ SEM. * P < 0.05. ** P < 0.01. *** P < 0.001.

cultures derived from pediatric patients (43). The results of these two studies suggest that further characterization of ALI cultures is required for full examination of the regulation and roles of E-cadherin and other barrier function proteins in asthma.

Consistent with previous data (43), we observed increased expression of Muc5AC and PAS staining in airway sections from patients with asthma. However, this did not translate to observable differences in ALI cultures. The reasons for this result are unknown. However, the ALI culture system is limited insofar as it is a model and does not represent the asthmatic airway environment, consisting of multiple cells and mediators that can influence epithelial functions. However, our findings are consistent with those of Parker and colleagues, who observed no differences in the expression of Muc5AC in ALI cultures derived from pediatric asthmatic and nonasthmatic donors (43). Interestingly, their study did show increased numbers of goblet cells in asthmatic-derived pediatric cultures by TEM. Overall, these findings suggest that other factors *in situ* within the asthmatic airway, such as the IL-13/IL-4 receptor α complex, EGFR, or neutrophil elastase, are important in driving the expression of Muc5AC (51–53). In support of this notion, our preliminary data (reported in abstract form) demonstrated that the addition of IL-13 and IL-4 to ALI media induces goblet-cell hyperplasia (54).

The airway epithelium is constantly exposed to a vast array of environmental challenges, including airborne allergens, pathogens, and potential toxic agents, and as such, it must respond effectively with regulated inflammatory responses. In the absence of environmental challenge, no differences in cytokine release were evident between asthmatic and nonasthmatic cultures, consistent with the findings of Parker and colleagues, who studied pediatric asthmatic ALI cultures and found no difference in the basal release of IL-6 and IL-8 (43). However, in response to infection with RSV and exposure to EHC-93, asthmatic ALI cultures displayed an exuberant inflammatory cytokine response compared with nonasthmatic ALI cultures. Several studies

reported that lung epithelial cell lines and primary bronchial epithelial cells can phagocytose ambient particles (EHC-93), both in culture and *in vivo* (55–58). The observation that IL-6, IL-8, IL-13, and GM-CSF are released by epithelial cells is consistent with previous studies *in vivo* and *in vitro* demonstrating that exposure to particulate matter induces a dose-dependent release of these cytokines (16, 35, 59, 60). The degree of oxidative stress and inflammation induced in the lung by particulate matter has been attributed to the chemical reactivity of specific constituents such as transition metals (Fe, Cu, Zn, Ni, and V) and organic compounds, especially polycyclic aromatic compounds (61–65). Furthermore, the cytotoxic and proinflammatory responses of lung epithelium were shown to vary substantially, depending on the source and the water-soluble and insoluble fractions of particulate matter used (35, 66, 67). Although the EHC-93 particulate matter used in this study was dissolved in a limited volume of 100 μ l in ALI cultures, we could not replicate an *in vivo* gaseous exposure in our model. However, ambient air, depending on location, can consist of a complex mixture of pollutants, and the impact of each of these constituents remains unresolved. Thus, although EHC-93 is a well-characterized source of urban particulate matter, it does not fully represent the potential exposure and responses of the airway epithelium to ambient pollution *in vivo*.

RSV is a single-stranded RNA virus of the *Paramyxoviridae* family, whose major site for replication *in vivo* is the airway epithelial cell (68). Previous studies of virus–epithelial cell interactions *in vitro* used both bronchial epithelial cell lines and ALI cultures to analyze the secretion of IL-6, IL-8, and GM-CSF (69, 70). To our knowledge, ours is the first study to demonstrate that these cytokines are elevated in response to RSV exposure in asthmatic-derived ALI cultures. In support of our data, elevated concentrations of IL-6, IL-8, and GM-CSF were previously shown to be elevated in bronchoalveolar lavage fluid from patients infected with RSV (71, 72). These mediators are innate inflammatory cytokines and chemoattractants that could

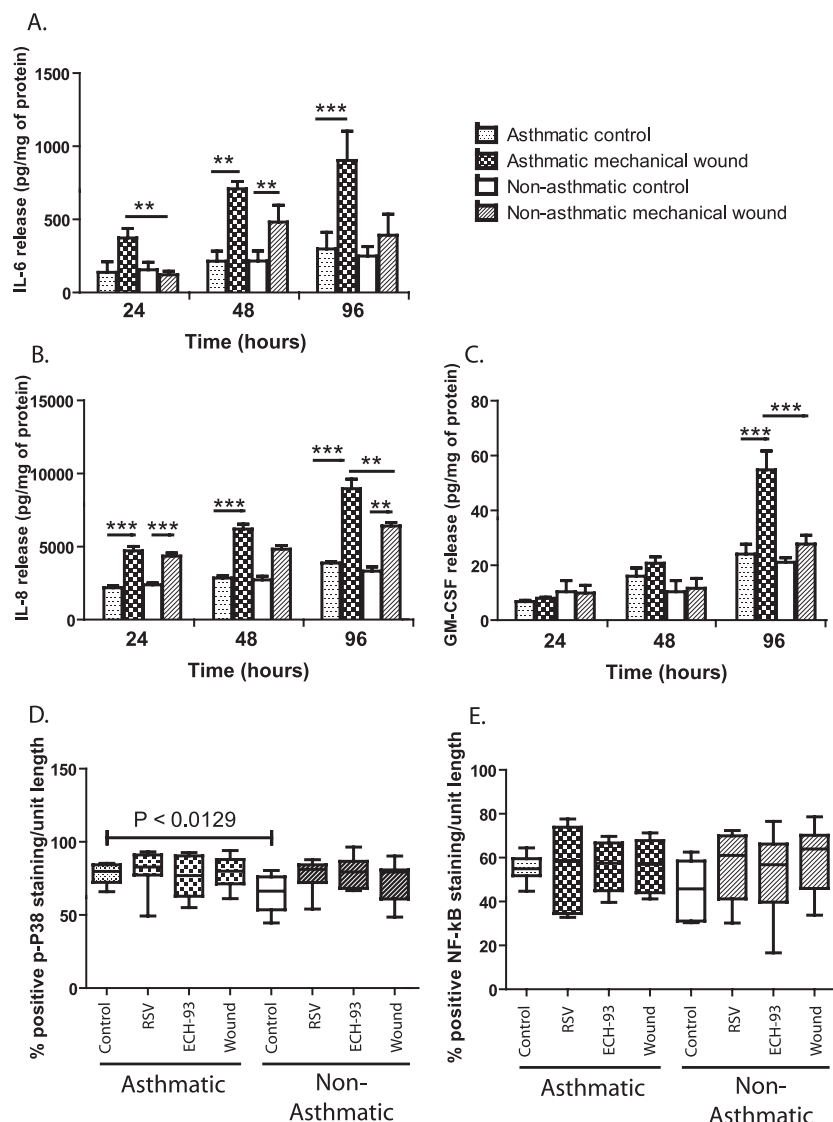


Figure 6. Asthmatic ALI cultures demonstrate an enhanced inflammatory response via p38-dependant mechanisms. Basolateral supernatants from asthmatic ALI cultures (dotted bars), wounded asthmatic ALI cultures (checked bars), nonasthmatic ALI cultures (open bars), and wounded nonasthmatic cultures (dashed bars) were collected at 24, 48, and 96 hours and analyzed by ELISA for the release of (A) IL-6, (B) IL-8, and (C) GM-CSF, measured as pg/mg of protein $\pm$ SEM. $**P < 0.01$. $***P < 0.001$. ALI sections from asthmatic and nonasthmatic donors were stained with phospho-Y182-p38 (D) or NF- κ B (E), and data are expressed as percentages of positive staining/unit length, quantified using Image Pro Plus software.

play an important role in the persistence of inflammation in asthma through the recruitment of neutrophils, eosinophils, basophils, and monocytes. Although we observed an elevated release of IL-6, IL-8, and GM-CSF from asthmatic compared with nonasthmatic-derived ALI cultures, we did not find elevated numbers of RSV-positive cells in asthmatic ALI cultures. Furthermore, the cells positive for RSV in both asthmatic and nonasthmatic-derived ALI cultures were found at the apical surface of the ALI culture. This finding is in line with previous studies by Mellow and colleagues (73) and Zhang and colleagues (74), which demonstrated that RSV infects ciliated airway epithelial cells. Although we observed the formation of cilia via TEM in asthmatic ALI cultures, an immunohistochemical analysis indicated that the majority of cells within asthmatic-derived cultures stained positive for cytokeratin-5, a basal cell marker. We and others previously demonstrated that submerged monolayer cultures of pulmonary epithelial cells, which are devoid of cilia, can be infected with RSV and produce a cytokine response (75, 76). In future studies, it will be of importance to determine the exact phenotype of cytokeratin-5-positive cells in asthmatic ALI cultures and their susceptibility to RSV infection. This study was also limited in that only the release of basolateral cytokines was measured. Although Mellow and colleagues (73) and Zhang and colleagues (74) previously demonstrated in nonasthmatic

ALI cultures that the secretion of cytokines is polarized to the basolateral supernatant after apical infection with RSV, the possibility remains that apical cytokine release may be altered in asthmatic ALI cultures. However, we were unable to answer this question due to a lack of reproducible cytokine measurements after washing the ALI apical surface.

To understand further the exuberant cytokine response generated by asthmatic epithelial cells, we mechanically wounded the ALI cultures to model a nonenvironmental insult, to determine if dysregulated inflammation occurred after any type of epithelial damage. Surprisingly, in response to wounding, we also observed an elevated release of IL-6, IL-8, and GM-CSF from asthmatic compared with nonasthmatic ALI cultures. Freishtat and colleagues also demonstrated an enhanced release of cytokines from asthmatic compared with nonasthmatic ALI cultures after wounding, although a different subset of cytokines was analyzed (TGF- β 1, IL-13, IL-1 β , IL-6, and IL-10) (77). Although we did observe an elevated release of IL-13 in both asthmatic and nonasthmatic donors at 24 hours after wounding, we did not observe an increased release of IL-13 at 48 hours after wounding, as demonstrated by Freishtat and colleagues (77). This difference may be attributable to the small number of donors, the limited clinical phenotyping, and particularly the atopic status of donors used in each study. In response to wounding,

asthmatic-derived ALI cultures demonstrated comparable wound closure in nonasthmatic patients after 96 hours, although asthmatic cultures exhibited delayed repair kinetics. This finding is consistent with the findings of Freishtat and colleagues, who also observed a delayed wound closure of asthmatic ALI cultures after 48 hours that could be modified by the addition of steroids, but that study did not involve later time points (77). In our study, two of the asthmatic donors were receiving corticosteroids, and one was receiving montelukast, before the isolation of their epithelial cells. However, the cells were maintained in culture for up to 50 days, which means that the responses we observed are likely to be intrinsic rather than steroid-dependant. Because of the size of our study cohort, we could not draw any direct comparisons between steroid and nonsteroid use and the responses of ALI-airway epithelial cells cultures.

Many of the signaling pathways activated by environmental stimuli, stress, and inflammatory cytokines converge on the activation of NF- κ B and p38 MAP kinase pathways in the airway epithelium (37–40). We found that the baseline expression of active p38 but not NF- κ B was greater in asthmatic ALI cultures compared with nonasthmatic cultures. This finding is consistent with a previous biopsy study demonstrating strong phospho-p38 staining, specifically in epithelial cells, in patients with mild and severe asthma compared with healthy control subjects (78). The activation of p38 was previously shown to be involved in the posttranscriptional regulation of RSV-induced cytokine expression (79). Similarly, particulate matter was demonstrated to induce IL-6 and IL-8 gene transcription via the activation of p38 in bronchial epithelial cells (80, 81). Immunostaining indicated that concentrations of phosphorylated p38 trended to increase after infection with RSV, exposure to EHC-93, and mechanical wounding, which may be due to limited sensitivity of the methodology used, but unfortunately the collection of cell lysates for analysis by ELISA was not possible because of the number of ALI cultures required. The finding of enhanced phospho-p38 staining in asthmatic epithelium before environmental challenge requires further investigation, as it may have implications for the initial exuberant inflammatory response observed in asthmatic-derived ALI cultures.

Because of the nature of asthmatic exacerbations, investigating the kinetics of acute inflammatory events within the airway epithelium is technically difficult, especially in response to specific exposures such as RSV and particulate matter. Although the use of pseudostratified ALI epithelial cultures has several advantages over monolayer cultures, including the preservation of epithelial architecture and multiple epithelial cell types, our study contains limitations. Firstly, this study only compared the airway epithelia of six asthmatic and six nonasthmatic donors. Although the use of donor lungs provides sufficient airway epithelial cells to obtain the data reported in this study, the histories of patients regarding atopic status and lung function were not available. Although all of the nonasthmatic donors included in this study had no comorbidities or current pulmonary diseases and had died of head trauma, this does not rule out the possibility that these individuals may have manifested an undiagnosed pulmonary disease. Secondly, the data are primarily focused on donors with fatal asthma and sub-basement membrane fibrosis. Future studies will need to include well-phenotyped patients with controlled mild and moderate asthma, for a full understanding of the role played by RSV and particulate matter in asthma exacerbations.

In conclusion, we demonstrate that ALI cultures can be used to investigate and compare many of the phenotypic differences within the airway epithelium of asthmatic and nonasthmatic patients. In addition, we report that asthmatic-derived ALI cultures respond with an increased expression of inflammatory mediators IL-6, IL-8, and GM-CSF to both environmental

(infection with RSV and exposure to EHC-93) and nonspecific mechanical damage, compared with nonasthmatic ALI cultures. The mechanisms involved in these innate differences within the asthmatic epithelium require further investigation, if we are to understand more fully the pathogenesis of asthma.

Author Disclosure: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors acknowledge the technical assistance of Anna Meredith at the James Hogg Research Centre of the University of British Columbia, with regard to the Luminex multiplex cytokine panel analysis, and Dr. David Walker and Dr. Fanny Chu for assistance with TEM. The authors also thank Dr. Renaud Vincent from Health Canada for making the EHC-93 available.

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